ATTACHMENT II – PROTOCOL

Ecolab
Study Identification Number 1200063

REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

STUDY TITLE: Aqualogic Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Virus: Rhinovirus Type 37 -260 ppm

EPA REG. NO.: 1677-

STUDY IDENTIFICATION NUMBER: 1200063

PROPOSED STUDY INITIATION/COMPLETION DATES

Initiation July 3, 2012

Completion August 30, 2012

DESCRIPTION OF STUDY OBJECTIVE

Aqualogic (EPA Registration No. 1677-) will be tested according to Ecolab Microbiological Services SOP Method MS505-07; *Virucidal Efficacy Assay for Hard Surfaces* to determine the virucidal efficacy against Rhinovirus Type 37 after a 30 second exposure time at ambient temperature when diluted to 0.0260% free available chlorine in 400 ppm synthetic hard water per the Confidential Statement of Formula (CSF). The actual dilutions which will be performed for the test substance use-solutions will be determined subsequent to the chemical quality verification to deliver the required level of active ingredient, and documented in the raw data. The test substance will be challenged by the addition of 5% fetal bovine serum to the test system. The test substance will be applied to the carriers at a distance of 6-8 inches for 3 sprays. ASTM E 1053 section 11 Water and Environmental Technology volume 11.05 2009 is the test method utilized in determining the virucidal efficacy against Rhinovirus Type 37.

Ecolab
Study Identification Number 1200063

TEST SUBSTANCE IDENTIFICATION

Test Substance Name: Aqualogic

Batch Identification

1. 051512DT
2. 052912DT

Both batches will be used to determine the use-solution chemical quality verification analysis.

An aliquot of the test substance will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, MN until the quality of the formula no longer affords evaluation. Test substance not dispersed for retention, chemical quality verification or efficacy testing will be stored in Ecolab Microbiological Services cabinet until disposed.

QUALITY ASSURANCE UNIT MONITORING

The protocol, chemical quality verification in-life, chemical quality verification data, pesticide efficacy in-life and final report are proposed to be inspected by the Ecolab Quality Assurance Unit (QAU) in accordance with their current standard operating procedures. The following proposed Ecolab QA inspections are for planning purposes only and may change. Ecolab QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in Ecolab QA inspections from those proposed below will not require revision of this protocol.

A. Proposed QAU Monitoring

Protocol Audit
Chemical Quality Verification In-Life Inspection
Chemical Quality Verification Data Audit
Pesticide Efficacy In-Life Inspection
Final Report Audit

CHEMICAL QUALITY VERIFICATION

A. Proposed Experimental Initiation/Termination Dates

Experimental Initiation Date: July 12, 2012

Experimental Termination Date: July 26, 2012

Ecolab
Study Identification Number 1200063

B. Method

Chemical analysis will be performed on each test substance concentrate batch to determine the concentration of the active ingredient under ECOLAB GLP study number 1200062. Chemical analysis will also be performed on the test substance use-solution. The use-solution preparation will be documented in the raw data.

If the test substance concentrate is at the lower certified limit, 0.0660% free available chlorine, and diluted at the same dilution ratio as used for the nominal values, then the resulting use-solution would have 260 ppm free available chlorine. The following calculation will used to determine the amount of test substance concentrate in a 100 g use-solution diluted to 260 ppm (or 0.0260%) free available chlorine:

$$\% \text{ Dilution} = \frac{(\text{nominal ppm}) \times (100) \times (100)}{(\% \text{ active on label of concentrate}) \times (10^6)}$$

$$\% \text{ Dilution} = \frac{(325) \times (100) \times (100)}{(0.0825) \times (10^6)} = 39.4\%$$

ppm if concentrate is at the Lower Certified Limit (LCL) =
(% LCL/100) X (% Dilution/100) X (specific gravity) X 10^6 =
ppm at LCL = (0.0660%/100) X (39.4%/100) (0.999) X 10^6 = 260 ppm

Amount of Test Substance needed to be at or below the LCL =

$$\frac{\text{ppm at LL} \times 100 \times \text{g amount of use-solution to be made}}{(\% \text{ active}) \ 10^6} = \text{grams of Test Substance}$$

In order to prepare the test substance use-solution using weight to weight measurements, the specific gravity was incorporated into the calculations resulting in 260 ppm (or 0.0260%) free available chlorine as the lower limit. The use-solution prepared for the test may be of any size, and amounts used to prepare the solution may be ± 0.03 g of the calculated value.

The chemical quality verification will be performed by the Analytical Lab using the method listed below. The method has been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution samples.

QATM-007; Available Chlorine
Available chlorine content is determined by reduction of the chlorine to chloride by iodide ion. The iodine liberated by this reaction is then determined by titration with sodium thiosulfate, either manually or potentiometrically with an automatic titrator.

The most current QATM will be used during the course of this study for the chemical and physical analysis.

Ecolab
Study Identification Number 1200063

C. Interpretation of Results

The concentration of the active ingredient in the test substance concentrate batches will be judged acceptable for pesticide efficacy testing if within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Free Available Chlorine*	0.0660%	0.1030%

*The equivalent weight of NaOCL (sodium hypochlorite) to the equivalent weight of Cl_2 (Chlorine) is 37.2/35.5 = 1.05. Dividing the sodium hypochlorite concentration by the ratio of the equivalent weight of sodium hypochlorite to the equivalent weight of chlorine results in the free available chlorine concentration.

The concentration of the active ingredients in the test substance use-solution diluted to 260 ppm will be judged acceptable for pesticide efficacy testing if within the acceptance limit of 0.0234 – 0.0286% available chlorine.

After diluting the test substance concentrate to the 260 ppm (0.0260%) free available chlorine, the nominal concentration of the active ingredient is <1.0%. Therefore, the Calculated Lower Acceptance Limit and Calculated Upper Acceptance Limit for available chlorine will be expanded to accommodate method variability and suitable rationale. The expanded ranges are based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

Calculated Lower Acceptance Limit for available chlorine
= [0.0260% - (0.0260 x 0.1)] = 0.0234%
Calculated Upper Acceptance Limit for available chlorine
= [0.0260% + (0.0260 x 0.1)] = 0.0286%

The chemical quality verification raw data will be reported in the final report of this study.

PESTICIDE EFFICACY TESTING

A. <u>Proposed</u> Experimental Start/Termination Dates

Experimental Start Date	July 12, 2012
Experimental Termination Date	July 19, 2012

Ecolab
Study Identification Number 1200063

B. Methods

Pesticide efficacy data will be generated by the Ecolab Microbiological Services Laboratory using the methods listed below. See the specific methods in the Protocol Appendix.

Method Number	Method Name
MS505-07	*Virucidal Efficacy Assay for Hard Surfaces*
MS500-07	*Cell Culture Procedure*
MS504-02	*Preparation of Sephadex Columns*
MS008-23*	*Synthetic Hard Water Preparation & Standardization*
MS088-18	*Test Substance Use-Solution Preparation for Analysis*

* MS008 will be followed with the following exception: Milli-Q Water or Lab Purified Water may be used in the preparation and/or titration of hard water.

Test Method Requirement and Test System Justification

Data from one test surface for each of two U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2200 Disinfectants for Use on Hard Surfaces –Efficacy Data Recommendations March 12, 2012. The test system for this study is Rhinovirus Type 37. ASTM method E 1053 for the above stated virus are recommended based on the U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2200 Disinfectants for Use on Hard Surfaces – Efficacy Data Recommendations March 12, 2012. Also, U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2000 General considerations for Public Health Uses of Antimicrobial Agents March 12, 2012 applies to this study.

A carrier method is used to generate supporting virological data. The virus is inoculated onto a glass surface (carrier), dried, exposed to the test substance for a specified exposure time, and assayed for viral infectivity.

Test Method Justification

Virucidal Effiacy testing will be performed according to Ecolab Microbiological Services SOP MS505-07 *Virucidal Efficacy Assay for Hard Surfaces*, which was created from ASTM method E 1053.

Ecolab
Study Identification Number 1200063

Virus

Rhinovirus Type 37, strain 151-1 ATCC VR-1147 that will be used in this study was obtained from the American Type Culture Collection, Manassas, VA. A portion of the stock culture was propagated, aliquoted, and stored at ≤ -70°C. On the day of testing, a vial or vials will be thawed from storage, pooled if necessary, and refrigerated until used in the test.

Organic Soil

5% Fetal Bovine Serum

Test Cell Cultures

The cell culture that will be used in this study, HeLa Cells ATCC CCL-2, was obtained from the American Type Culture Collection, Manassas, VA. The cell culture was propagated, aliquoted, and stored in liquid nitrogen. The cell culture is maintained and used at the appropriate cell density in tissue culture laboratory products at 35 ± 2°C in a humidified atmosphere at 5 ± 2% CO_2.

The growth medium to be used in the study to propagate the test cell culture is Minimum Essential Medium, Eagle (EMEM) with Earle's Balanced Salt Solution and Non-Essential Amino Acids supplemented with 1-10% (v/v) heat inactivated fetal bovine serum. The medium may be supplemented with any of the following: 50 - 100 IU penicillin and 50 - 100 µg/mL streptomycin.

HeLa Cells, from the third or greater transfer, will be prepared in 24 well assay plates prior to the test. The cultures will incubate at 35 ± 2°C in a humidified atmosphere at 5 ± 2% CO_2 for one to two days. The appropriate media will be used to renew the cell cultures prior to the start of the test.

Statement of Proposed Statistical Method

None

Test Substance Diluent

Sterile Synthetic Hard Water at 400 ppm (as $CaCO_3$) prepared as described in Ecolab Microbiological Services SOP MS008-23; *Synthetic Hard Water Preparation & Standardization* will be the diluent.

Ecolab
Study Identification Number 1200063

Test Substance Concentration

Antimicrobial efficacy testing will be performed with **Aqualogic** diluted to 260 ppm free available chlorine.

Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Available Chlorine	0.0660%	0.1030%

The dilution procedure is based on results of the Chemical Quality Verification study. To achieve dilution of the test substance concentrate to the lower limit of available chlorine, the test substance use-solution will be prepared based on the available chlorine results and documented in the raw data. The following calculation will be used to determine the dilution procedure for each test substance batch to result in 260 ppm of available chlorine.

$$\% \text{ Dilution} = \frac{(\text{nominal ppm}) \text{ X } (100) \text{ X } (100)}{(\% \text{ active on label of concentrate}) \text{ X } (10^6)}$$

$$\% \text{ Dilution} = \frac{(325) \text{ X } (100) \text{ X } (100}{(0.0825) \text{ X } (10^6)} = 39.4\%$$

ppm if concentrate is at the Lower Certified Limit (LCL) =
(% LCL/100) X (% Dilution/100) X (specific gravity) X 10^6 =
ppm at LCL = (0.0660%/100) X (39.4%/100) (0.999) X 10^6 = 260 ppm

Amount of Test Substance needed to be at or below the LCL =

$$\frac{\text{ppm at LL X 100 X g amount of use-solution to be made}}{(\% \text{ active}) \ 10^6} = \text{grams of Test Substance}$$

The use-solution prepared for the test may be of any size, and amounts used to prepare the solution may be ± 0.03 g of the calculated value.

Test Surface

Glass petri dishes, 100 x 15 mm

Spray Distance and Number of Trigger Pulls

The diluted test substance will be applied to the virus by spraying from a spray bottle at a distance 6 to 8 inches with 3 trigger pulls.

Exposure Time/Temperature

The test systems will be exposed to the test substance for 3 minutes at ambient temperature (15 – 30 °C).

Ecolab
Study Identification Number 1200063

Neutralizer

Sephadex columns with Fetal Bovine Serum as diluent for 10^{-2} dilution, or GE Sephacryl columns with Fetal Bovine Serum as diluent for 10^{-2} dilution. Neutralizer used will be specified on the benchsheets.

Test Medium

Minimum Essential Medium, Eagle (EMEM) with Earle's Balanced Salt Solution and Non-Essential Amino Acids supplemented with 1-10% (v/v) heat inactivated fetal bovine serum. The medium may be supplemented with any of the following: 50 - 100 IU penicillin and 50 – 100 µg/mL streptomycin.

The media for the infectivity assay and control cultures may be renewed periodically during incubation.

Incubation Time/Temperature

Infectivity assayed cell cultures and controls are incubated for 7 to 10 days at 35 ± 2°C in a humidified atmosphere at 5 ± 2% CO_2. The cultures will be evaluated periodically during the incubation period for the absence or presence of cytopathic effect (CPE), cytotoxcity, and viability.

Test Controls

The following controls will be incorporated with the test procedure:

(a) Dried virus film recovery –four determinations per dilution assayed
(b) Virus stock titer confirmation –four determinations per dilution assayed
(c) Test substance cytotoxicity –four determinations per dilution assayed
(d) Neutralization verification –four determinations per dilution assayed
(e) Viability of cell culture controls –four determinations per assay plate

Details on a through e of the above controls can be found in Ecolab SOP MS505-07 located in the Protocol Appendix.

Two dried virus film controls will be performed. The $TCID_{50}$ values will be averaged for use in the log reduction calculations for the test substance. The following equation will be used:

$$\frac{(TCID_{50} \text{ of film replicate 1} + TCID_{50} \text{ of film replicate 2})}{2} = \text{Average } TCID_{50} \text{ dried virus film control}$$

Ecolab
Study Identification Number 1200063

Interpretation of Test Results

A valid test requires a viral titer of 10^4 particles to be recovered from the tested surface. To obtain a virucidal claim, the test substance must demonstrate complete inactivation of the virus at all dilutions when no cytotoxicity is present or at all dilutions higher than the cytotoxic level. The test substance must demonstrate a log reduction in viral titer of greater than or equal to 3 for both batches when cytotoxicity is present. (EPA Product Performance Guidelines 810.2200 Disinfectants) Any dilutions showing virucidal activity of the test substance in the neutralizer control assay will not be considered in determining the reduction of infectivity by the test substance. All cells in the viability of cell culture control wells must remain viable for the test to be valid.

DATA RETENTION

Following the completion of the study, the original raw data and an exact copy of the final report will be archived at the Ecolab Schuman Campus in Eagan, Minnesota or at an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

Ecolab
Study Identification Number 1200063

- **Name and Address of Sponsor**

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  Eagan, MN 55121

- **Name and Address of Testing Facility**

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  Eagan, MN 55121

- **Name of Study Director**

  Lisa Hellickson
  Ecolab Schuman Campus
  655 Lone Oak Drive
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Sponsor — Date 7/3/12

Study Director — Date 7/3/12

Ecolab
Study Identification Number 1200063

PROTOCOL APPENDIX

Microbiological Services (MS) Methods:

MS505-07	*Virucidal Efficacy Assay for Hard Surfaces*	Pages 1-10
MS500-07	*Cell Culture Procedures*	Pages 1-6
MS504-02	*Preparation of Sephadex Columns*	Pages 1-3
MS008-23	*Synthetic Hard Water Preparation & Standardization*	Pages 1-5
MS088-18	*Test Substance Use-Solution Preparation For Analysis*	Pages 1-6

ECOLAB
MICROBIOLOGICAL SERVICES

TITLE: **Virucidal Efficacy Assay for Hard Surfaces**

NUMBER: **MS505-07**

EFFECTIVE: **06/01/12**

1.0 PURPOSE

To describe the evaluation of liquid antimicrobial solutions for virucidal efficacy on inanimate non-porous environmental surfaces.

2.0 EQUIPMENT

2.1 Water Bath
2.2 Biological Safety Cabinet (BSC)
2.3 Inverted Microscope
2.4 Centrifuge
2.5 CO_2 Incubator
2.6 Pipette Filling Device (e.g. Pipetboy)
2.7 Micropipettors
2.8 Humidity Chamber

3.0 MATERIALS

3.1 70% Alcohol
3.2 Seeded Cell Culture Plates
3.3 Cell Culture Media
3.4 Fetal Bovine Serum
3.5 Sephadex Gel
3.6 Virus Stock
3.7 Serological Pipets
3.8 Pipette Tips
3.9 50 mL Conical Centrifuge Tubes
3.10 Sterile Glass Petri Dishes, 100 × 15 mm
3.11 Cell Scrapers
3.12 Snap cap tubes (or similar)
3.13 GE Sephacryl Columns S-400

4.0 SAFETY

4.1 All viruses cultured for virucidal efficacy testing are classified at Biosafety Level 2 and most are capable of causing infections in humans. Use required personal protective equipment and follow established lab safety procedures (refer to MS080).

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TITLE: Virucidal Efficacy Assay for Hard Surfaces

NUMBER: MS505-07

5.0 CELL CULTURES

5.1 Prior to the day of testing, cell culture plates must be prepared (refer to MS500). Alternately, primary cell culture plates may be ordered from a reputable supplier.

5.2 Microscopically examine the cell culture plates to be used in testing to ensure they display the proper integrity and confluence required for the growth of the test virus.

5.3 Prior to inoculation, change the cell culture media in each cell culture plate to be used in testing.

5.3.1 Aspirate the spent media from each well in the cell culture plate.

5.3.2 Add 1.0 mL of pre-warmed media, appropriate for the growth of the test virus, to each well.

5.4 Re-incubate the plates at 35 ± 2°C and 5 ± 2% CO_2 until use.

5.5 Four wells on each 24 well plate should remain uninoculated as a cell culture viability control.

6.0 NEUTRALIZATION COLUMNS

6.1 Sephadex Gel Columns

6.1.1 Prior to the start of testing, centrifuge the conical tube containing the column at 2500 rpm for three minutes.

6.1.2 Post-centrifugation, the column bed size should be approximately between 9.5 mL and 11.0 mL. Record the range of the bed sizes on Form 3141.

6.1.2.1 If centrifuging the columns in batches select and mark the two columns with the highest and lowest bed sizes and centrifuge these two in the same batch. These will provide the post-centrifugation bed size range. Ensure all columns actually fall within this range.

6.1.3 Place each column in a new 50 mL conical tube for use in testing.

6.2 GE Sephacryl Columns

6.2.1 Resuspend the resin in the column by vortexing
6.2.2 Loosen the cap one-quarter turn and snap off the bottom closure
6.2.3 Place the column in a collection tube

6.2.4 Pre-spin the column for one minute at 700 × g
6.2.5 Transfer the column to a new clean microcentrifuge tube

6.3 Fetal Bovine Serum (FBS)

6.3.1 Refer to 12.2.3 for instructions.

7.0 STOCK VIRUS

7.1 Thaw the required number of vials at 2 – 8°C, room temperature, or in a 35 ± 2°C water bath (ensure the vials are kept in the water bath only until just thawed). Virus must be thawed the day of testing.

7.2 Combine multiple vials into one snap-cap or conical tube and mix well.

7.3 Preparation of low titer virus stock for neutralization controls:

7.3.1 The quantity of virus stock required to confirm the virus stock titer and to prepare the low titer virus stock should be removed prior to the addition of any additional FBS or Sodium Stearate for soil loads.

7.3.2 Refer to Form 3133 for the lot number of virus stock to be used in testing for the viral titer ($TCID_{50}$/0.1 mL) of that virus stock.

7.3.3 Prepare a $10^{3.5} – 10^{4.5}$ viral titer stock (referred to as the low titer virus stock) by making the appropriate 10-fold serial dilutions.

Note: The final titer will become $10^{2.5} – 10^{3.5}$ after addition to cell cultures.

7.3.4 Ensure a sufficient quantity of the low titer virus stock is prepared. One 24-well neutralization plate requires 3.0 mL.

7.3.5 The low titer virus stock should be stored at 2 – 8°C until use.

7.4 If soil load is requested, the virus stock suspension most likely contains 5% FBS. If the addition of soap scum is also requested, the virus stock should contain 5.3% FBS.

7.4.1 If the virus stock suspension contains less FBS than is needed, add the appropriate amount of FBS to increase the concentration.

Calculation

$$\text{Amount of FBS Required (mL)} = \left| \frac{\text{Desired FBS\%}}{100} \times \text{Volume of Virus Stock} \right| - \left| \frac{\text{FBS\% Present}}{100} \times \text{Volume of Virus Stock} \right|$$

Example: 2.0 mL virus stock contains 2% FBS; 5% FBS is required.

$$\text{Amount of FBS Required (mL)} = \left| \frac{5\%}{100} \times 2.0\text{ mL} \right| - \left| \frac{2\%}{100} \times 2.0\text{ mL} \right|$$

$$= \left| 0.05 \times 2.0\text{ mL} \right| - \left| 0.02 \times 2.0\text{ mL} \right|$$

$$= \left| 0.1\text{ mL} \right| - \left| 0.04\text{ mL} \right|$$

$$= 0.06\text{ mL}$$

Result: 0.06 mL FBS should be added to 1.94 mL virus suspension.

7.4.2 *Addition of Sodium Stearate (Soap Scum)*

7.4.2.1 Add the appropriate amount of FBS to increase the concentration to 5.3% (refer to 7.4.1).

7.4.2.2 Add the calculated amount of 0.1% Sodium Stearate to a volume of the virus stock to yield a suspension containing 0.005% Sodium Stearate.

Calculation

$$\text{Amount of 0.1\% Sodium Stearate Required (mL)} = \frac{0.005\%}{0.1\%} \times \text{Volume of Virus Stock}$$

Example: Need to add Sodium Stearate to 6.0 mL virus stock.

$$\text{Amount of 0.1\% Sodium Stearate Required (mL)} = \frac{0.005\%}{0.1\%} \times 6.0\text{ mL}$$

$$= 0.05 \times 6.0\text{ mL}$$

$$= 0.3\text{ mL}$$

Result: 0.3 mL Sodium Stearate should be added to 5.7 mL of virus stock

7.5 The virus stock should be kept on ice and/or refrigerated (2 – 8°C) for the duration of testing.

7.6 Any virus remaining after testing will be discarded.

TITLE: Virucidal Efficacy Assay for Hard Surfaces

NUMBER: MS505-07

8.0 TEST SUBSTANCE(S)

8.1 Confirm the physical description of the test substance concentrate matches the description provided.

8.2 If the test substance requires dilution, ensure the quantity of test substance diluted is equal to or greater than 1.0 mL or 1.0 g.

8.3 The use solution must be tested within three hours of preparation unless otherwise instructed.

8.4 Refer to MS088 for further instructions.

8.5 If synthetic hard water is to be used as the diluent, refer to MS008 for preparation instructions.

9.0 VIRUS STOCK TITER CONFIRMATION

9.1 The virus stock will be assayed to ensure the titer has not significantly declined.

9.2 Prepare the appropriate 10-fold serial dilutions and refer to section 13.0.

10.0 DRIED VIRUS FILM (CARRIER) PREPARATION

10.1 Mix virus suspension thoroughly.

10.2 Place 200 µL of the virus suspension onto the bottom of a sterile glass 100 mm × 15 mm petri dish.

10.3 Spread the suspension to within approximately ¼" of the perimeter of the plate. Do not use the plate for testing if the suspension runs into the corner of the plate.

10.4 Allow the virus films to dry (with plate lids cracked) in the BSC (15 – 30°C) for 20 minutes or until visually dry.

10.5 Virus films may be dried (with plate lids cracked) in an environmental humidity chamber at 15 – 30°C for 20 minutes or until visibly dry. Record the temperature and humidity of the chamber.

11.0 TEST SUBSTANCE APPLICATION

11.1 Dried Virus Film Recovery Control

11.1.1 Add 2.0 mL of test medium to a dried virus film. Ensure the medium completely covers the bottom of the plate.

11.1.2 Allow the plate to stand in the BSC (15 – 30°C) for the same exposure time as requested for the test substances.

11.2 Product Tests

11.2.1 Add 2.0 mL of test substance to a dried virus film. Ensure the test substance completely covers the bottom of the plate.

11.2.2 Alternately, the test substance may be sprayed on to the virus film. Record the spray distance, time and/or number of trigger pulls. Ensure the test substance completely covers the bottom of the plate. Also, record the average weight of test substance sprayed by spraying an empty petri dish on a balance. Perform and record the weights of five replicates and average.

11.2.3 Alternately, the test substance may be applied by wiping with a saturated towelette. The towelette should be handled with sterile gloves and/or sterile forceps. The wiping procedure should be noted.

11.2.4 Allow the plate to stand in the BSC (15 – 30°C) for the requested exposure time.

12.0 END OF EXPOSURE PERIOD/NEUTRALIZATION

12.1 Just prior to the end of the exposure period, scrape the plate with a cell scraper to re-suspend the virus film. This is the 10^{-1} dilution of the virus.

12.2 At the end of the exposure period, immediately neutralize the suspension.

12.2.1 If using Sephadex columns for neutralization, pass the suspension through a previously prepared Sephadex gel column (refer to 6.1) using the syringe plunger. This remains the 10^{-1} dilution of the virus.

12.2.2 If using GE Sephacryl columns for neutralization, add 100 µL of the suspension to each of five previously prepared Sephacryl columns. Centrifuge the columns for two minutes at 700 × g. These remain 10^{-1} dilutions of the virus.

12.2.3 If using FBS for neutralization (alone or in conjunction with a column), add 100 µL of the suspension to 900 µL of FBS and mix well. This is the 10^{-2} dilution of the virus.

12.3 Following neutralization, make the appropriate 10-fold serial dilutions.

TITLE: Virucidal Efficacy Assay for Hard Surfaces

NUMBER: MS505-07

13.0 VIRUS RECOVERY

13.1 Inoculate 100 µL of each test dilution into the cell culture plates in quadruplicate.

13.2 Incubate the cell culture plates at 35 ± 2°C with 5 ± 2% CO_2 for seven to ten days.

13.3 It may be necessary to periodically refresh the media in the cell culture plates during the incubation time.

14.0 CYTOTOXICITY & NEUTRALIZATION CONTROLS

14.1 If Sephadex gel columns are being used for neutralization, pass 2.0 mL of the test substance through a previously prepared Sephadex gel column (refer to section 6.0) using the syringe plunger. This is the 10^{-1} dilution of the test substance.

14.2 If GE Sephacryl columns are being used for neutralization, pass 100 µL of the test substance through each of five previously prepared columns as in 12.2.2. These are 10^{-1} dilutions of the test substance.

14.3 If FBS was used (along or in conjunction with a column) add 100 µL of the test substance (after passing through column if necessary) to 900 µL of FBS and mix well. This is the 10^{-2} dilution of the test substance.

14.4 Make the appropriate 10-fold serial dilutions.

14.5 Cytotoxicity controls

14.5.1 Inoculate 100 µL of each cytotoxicity control dilution into the cell culture plates in quadruplicate.

14.6 Neutralization controls

14.6.1 Inoculate 100 µL of each cytotoxicity control dilution into the cell culture plates in quadruplicate.

14.6.2 To each of the inoculated cell culture plate wells, add 100 µL of the low-titer virus stock (refer to 7.3).

14.7 Incubate the cell culture plates at 35 ± 2°C with 5 ± 2% CO_2 for 7 – 10 days.

15.0 CELL CULTURE EVALUATION

15.1 The cell culture plates should be observed two to three times over the incubation period and evaluated for viral cytopathic effect (CPE) and cytotoxicity.

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TITLE: Virucidal Efficacy Assay for Hard Surfaces

NUMBER: MS505-07

15.1.1 CPE is graded based on the approximate percentage of cells infected; cytotoxicity is graded based on the approximate percentage of cells affected:

- 0 = negative for the presence of CPE; no cytotoxicity present
- C = cell changes noted upon initial evaluation that may or may not be caused by viral infection or cytotoxicity and further incubation is required
- 1 = CPE present; no more than 25% of the cells are infected
- 2 = CPE present; 25 – 50% of the cells are infected
- 3 = CPE present; 50 – 75% of the cells are infected
- 4 = CPE present; 75 – 100% of the cells are infected
- 1T = no more than 25% of the cells are cytotoxic
- 2T = approximately 25 – 50% of the cells are cytotoxic
- 3T = approximately 50 – 75% of the cells are cytotoxic
- 4T = approximately 75 – 100% of the cells are cytotoxic

Note: For reporting purposes, scores of 1T and 2T will be reported as 0; 3T and 4T as T.

16.0 $TCID_{50}$ AND VIRAL TITER REDUCTION CALCULATIONS

16.1 $TCID_{50}$ is the dilution of a virus needed to infect 50% of the number of inoculated cell cultures. The equation below is based on the Spearman Käber method.

$$\text{Log}_{10} \text{ of the reciprocal of the highest dilution showing CPE in all wells tested} - 0.5 + \frac{\text{Starting with the highest dilution showing CPE in all wells tested, the total number of wells showing CPE}}{\text{Number of test wells per dilution}} = \text{Log}_{10}\ TCID_{50}$$

16.2 Examples

Example 1

Dilution	Results
10^{-5}	++++
10^{-6}	++++
10^{-7}	0+0+
10^{-8}	0000
10^{-9}	0000

Example 2

Dilution	Results
10^{-5}	++++
10^{-6}	++++
10^{-7}	++0+
10^{-8}	0+00
10^{-9}	0000

+ = viral CPE present
0 = viral CPE absent

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16.2.1 No calculations are necessary for Example 1. The 10^{-7} dilution is the 50% end-point dilution as this is the highest dilution showing CPE and two of the four wells at this dilution are positive for the presence of virus. Therefore, the $TCID_{50}$ for this example is 10^{7}.

16.2.2 As there is no clear 50% end-point dilution for Example 2, the $TCID_{50}$ *must be calculated using the calculation above.*

- The highest dilution showing CPE in all wells tested is 10^{-6}
- The $\log_{10}$ of the reciprocal of 10^{-6} is 6.
- Starting with the highest dilution showing CPE in all wells tested, the total number of wells showing CPE counted is 8 (10^{-6} has four wells showing CPE, 10^{-7} has three and 10^{-8} has one, for a total of 8).
- The number of test wells per dilution is 4.

6	-	0.5	+	$\frac{8}{4}$	=	Log_{10} $TCID_{50}$
5.5			+	2	=	7.5

Result: The $TCID_{50}$ for this example is 7.5 $\log_{10}$ or $10^{7.5}$.

16.3 Once the $TCID_{50}$ for the virus control and the test replicates have each been calculated, subtract the test replicate $TCID_{50}$ from the virus control $TCID_{50}$ to obtain the log reduction in viral titer.

Note: If there are multiple virus control replicates, calculate the average $TCID_{50}$ for the controls. Subtract the test replicate $TCID_{50}$ from the average virus control $TCID_{50}$ to obtain the log reduction in viral titer.

17.0 TEST CRITERIA & EVALUATION OF RESULTS

17.1 US EPA

17.1.1 A titer of 10^{4} or greater must be recovered from the virus control.

17.1.2 If cytotoxicity is present, at least a three log reduction in viral titer must be shown beyond the level of cytotoxicity.

17.1.3 An efficacious product will demonstrate complete viral inactivation at all dilutions tested.

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TITLE: Virucidal Efficacy Assay for Hard Surfaces

NUMBER: MS505-07

17.2 Health Canada

17.2.1 The virus control must have a titer of at least four logs higher than the level displayed by the test substance.

17.2.2 An efficacious product will demonstrate a least a three log reduction in viral titer beyond the level of cytotoxicity in each of the carriers tested.

18.0 RELATED FORMS

18.1 Form 3141: Virucidal Efficacy Assay for Hard Surfaces

19.0 REFERENCES

19.1 ASTM E 1053 – Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces
19.2 US EPA DIS/TSS-7 / Nov. 12, 1981. *Efficacy Stat Requirements: Virucides*
19.3 National Standard of Canada: CAN/CGSB-2.161-97 – Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices
19.4 Freshney, R. I., (2005). Culture of Animal Cells: A Manual of Basic Technique. (5th ed.). Hoboken, NJ: John Wiley & Sons, Inc.
19.5 Mahy, B. W. and H. O. Kangro, (1996). Virology Methods Manual. London, England: Academic Press Limited.
19.6 Schmidt, N.J. and R.W. Emmons, (1989). Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections. (6th ed.). Washington, DC: American Public Health Association
19.7 MS008: Synthetic Hard Water Preparation & Standardization
19.8 MS080: Lab Safety & Environmental Control
19.9 MS088: Test Substance Use-Solution Preparation for Analysis

20.0 MOST RECENT REVISION SUMMARY

Added a new 11.2.3 to add a wiping application of the test substance.

Prepared by: _____ Date: 5·21·12

Quality Assurance: _____ Date: 22 MAY 2012

Management: _____ Date: 22 May 2012

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LPH 6/30/12

5

ECOLAB
MICROBIOLOGICAL SERVICES

Standard Operating Procedure

Ecolab Controlled Document

TITLE: **Cell Culture Procedure**

NUMBER: **MS500-07**

EFFECTIVE: **05/01/11**

1.0 PURPOSE

To describe the culture of mammalian cell lines for use in virucidal efficacy testing.

2.0 EQUIPMENT

2.1 Water Bath
2.2 Biological Safety Cabinet
2.3 CO_2 Incubator
2.4 Inverted Microscope
2.5 Centrifuge
2.6 Pipette Filling Device (e.g. Pipetboy)
2.7 Micropipettor
2.8 Liquid Nitrogen Storage Tank
2.9 Mr. Frosty

3.0 MATERIALS

3.1 70% Alcohol
3.2 Phosphate Buffered Saline (PBS), 1X
3.3 Trypsin Solution
3.4 Cell Culture Media
3.5 Serological Pipets
3.6 Cell Culture Flasks
3.7 50 mL Conical Centrifuge Tubes
3.8 Pipette Tips
3.9 Hemocytometer & Coverslip
3.10 Cryovials
3.11 Isopropanol (≥ 98%)

4.0 ASEPTIC TECHNIQUE

4.1 Aseptic technique must be practiced throughout every cell culture procedure.

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TITLE: Cell Culture Procedure

NUMBER: MS500-07

5.0 GENERAL INFORMATION

5.1 The following table lists suggested reagent volumes for different sized cell culture flasks:

Flask Size	Culture Medium	PBS Wash	Trypsin
T-25	5.0 mL	3 - 5 mL	0.5 - 1.0 mL
T-75	10 - 15 mL	5 - 10 mL	1.0 - 1.5 mL
T-150	20 - 30 mL	8 - 12 mL	2.0 mL

5.2 Growth media, also called plant media, usually contains 10% serum and should be used when cells are initially recovered from liquid nitrogen and when cells are passed. Refer to MS502 for the growth media types appropriate for specific cell lines.

5.3 After two to three days of growth, cells may be switched to maintenance media, which usually contains 2-5% serum. Refer to MS502 for the maintenance media types appropriate for specific cell lines.

5.4 Cell culture media should be warmed before use, unless otherwise noted. A 35 ± 2°C water bath or incubator may be used. Ensure unused media is removed from the water bath or incubator at the end of the day.

5.5 Growth of cells in a cell culture flask is measured by estimating the confluency of the cell monolayer. The confluency is the approximate percent of the flask's surface area that is covered by attached cells.

Example: A flask of cells is observed under the microscope and in each field observed, approximately half of the surface area is covered by cells, and the other half is bare plastic. The confluency for this flask of cells would be 50%.

5.6 To limit the effects subculturing may have on a cell line and thus ensure the cell line's integrity remains consistent throughout its use, a cell line will not be passed more than 15 times after it has been recovered from frozen. Passing a vial of frozen cells to a cell culture flask will not be included in the 15 passage limit. The number of passages from frozen will be documented on Form 3129.

6.0 RECEIPT OF CELL CULTURE STOCKS

6.1 Receipt of cell culture stocks from outside vendors (e.g. ATCC) will be documented on Form 3128.

6.2 Information accompanying the cells (e.g. product information sheets) will be stored in the Virology Lab Receipt binder.

TITLE: Cell Culture Procedure

NUMBER: MS500-07

7.0 CRYOPRESERVATION OF CELL LINES

7.1 Document these activities on Form 3129 and Form 3131.

7.2 The lot number from the culture collection agency will be used to track cultures along with the date prepared for storage.

7.3 Obtain a flask of cells to be cryopreserved. The monolayer should be at least 50% confluent.

7.4 Remove the cells with trypsin (refer to section 10.0) and resuspend in cold (2 – 8°C) cell culture medium.

7.5 Perform a cell count with a hemocytometer and use Form 3131 to calculate the volume of Cryopreservation medium required to resuspend the cell pellet to result in a final cell concentration of 1 x 10^6 to 5 x 10^6 cell per mL.

7.6 Centrifuge the cells at 200 x g for 5 - 10 minutes.

7.7 Slowly add the calculated volume of cryopreservation medium to the cell pellet. DMSO solutions must be added very slowly because the latent heat that is released during mixing can cause cell injury and death.

7.8 Label sterile cryovials with the identity of the cell line, the date prepared for storage and the passage number (if applicable).

7.9 Aliquot one mL amounts of the cell suspension into the cryovials and tighten the caps securely.

7.10 Fill a Mr. Frosty with approximately 250 mL of Isopropanol.

7.11 Place the cryovials into the Mr. Frosty and place at ≤ 70°C for at least 12 hours.

7.12 Remove the vials from ≤ -70°C and immediately place into liquid nitrogen for long-term storage.

7.12.1 The storage racks are numbered on the liquid nitrogen tank.

7.12.2 Each storage rack holds eight boxes which will be designated numbers one to eight, from top to bottom.

7.12.3 One or two cell lines may be stored in one box.

TITLE: Cell Culture Procedure

NUMBER: MS500-07

8.0 RECOVERY OF FROZEN CELLS

Note: The following is a general procedure for recovering frozen cells. The information received with the cells should be reviewed for additional recovery information.

8.1 Document these activities on Form 3129.

8.2 Add the appropriate volume of pre-warmed growth medium to a labeled T-25 or T-75 flask.

8.3 Remove the vial of cells from liquid nitrogen storage.

8.4 Thaw the vial of cells rapidly by gently swirling in a 35 ± 2°C waterbath. Keep the O-ring and cap out of the water bath to avoid possible contamination. Remove the cells as soon as they are thawed.

8.5 Transfer the entire contents of the vial to the T-25 or T-75 flask.

8.6 Gently rock the flask to distribute the cells evenly over the bottom of the flask.

8.7 Incubate the flask at 35 ± 2°C, 5 ± 2% CO_2 overnight to allow the cells to attach.

8.8 After the overnight incubation, remove the spent medium from the cells and add the appropriate volume of fresh growth medium.

9.0 CHANGING MEDIA

9.1 Media may be changed approximately every three to five days or if media becomes acidic (media color changes to yellow or yellow-orange) or alkaline (media color changes to hot pink/purple).

9.2 Document these activities on Form 3129.

9.3 Remove the spent medium from the cells, and add the appropriate volume of fresh medium to cell culture flask.

9.4 Return flasks to a 35 ± 2°C, 5 ± 2% CO_2 incubator.

10.0 PASSAGE OF CELL CULTURE MONOLAYERS

10.1 Document these activities on Form 3129.

10.2 Thaw an aliquot of trypsin at room temperature or in a 35 ± 2°C waterbath or incubator. Do NOT leave the trypsin at 35 ± 2°C for more than 20 minutes.

10.3 Remove the spent medium from the cells.

TITLE: Cell Culture Procedure

NUMBER: MS500-07

10.4 Rinse the cell monolayer twice with PBS and remove.

10.5 Add the appropriate volume of trypsin.

10.6 Rock the flask to ensure that the entire monolayer is covered with trypsin.

10.7 Incubate the flask at 35 ± 2°C for 1 - 5 minutes to allow the cells to detach from the surface. Gently tapping the flask against the heel of your hand will aid in the removal of adherent cells. Do NOT cause excessive foaming as this will damage the cells.

10.7.1 If the cells appear to be resistant to trypsinization, allow the cells to incubate longer in the trypsin with occasional tapping. Extremely resistant cells may require up to 30 minutes detaching from the surface. If this occurs, remove the cells that have come off the plastic within the first few minutes and neutralize them. Add more trypsin to the remaining cells. This may help to lower cell death due to excessive trypsinization and increase plating efficiency.

10.8 Neutralize the trypsin by adding growth cell culture medium appropriate for the cell line. The amount of medium added is dependent on the dilution required for passing the cells, but should be at least twice the volume of trypsin used.

10.9 Gently pipette up and down, while rinsing the bottom of the flask, to obtain a homogenous cell suspension.

10.10 Label new cell culture flasks with the cell type, lot number, passage number, passage ratio, date of passage and initials and add the appropriate volume of fresh cell culture media to each.

10.11 Transfer the required volume of the cell suspension to each new cell culture flask.

10.12 Gently rock the flask to distribute the cells evenly over the bottom of the flask.

10.13 Place flask in a 35 ± 2°C, 5 ± 2% CO_2 incubator.

10.14 During cell line passages, observe the cells for changes that might indicate microbial contamination. Document these observations on Form 3129.

10.14.1 Microbial contamination can be indicated by:

- Sudden changes in pH, indicated by a change in media color (usually red to yellow)
- Cloudiness in cell culture medium, or a film or scum on the surface
- Growth of bacteria, yeast and mold will be visible microscopically. Significant growth may be visible macroscopically
- Cell monolayer may become cytotoxic and deteriorate

TITLE: Cell Culture Procedure

NUMBER: MS500-07

10.15 Refer to Culture of Animal Cells: A Manual of Basic Techniques for more information on cell types and contamination if needed.

11.0 SEEDING 24-WELL PLATES

11.1 Document these activities on Form 3129.

11.2 Follow steps 10.2 – 10.9.

11.3 Dilute the cell suspension such that a 24 or 48 hour culture, depending on the cvell line, will yield between 50 and 90% confluency in the wells.

11.3.1 For most cell lines, dilute the cell suspension obtained from 10.9 1:10 in cell culture media. This will yield the appropriate cell concentration needed.

11.4 Dispense 1.0 mL of the diluted cell suspension into the wells of a 24-well plate. Gently mix the cell suspension between seeding each plate.

11.5 Place labeled plates in a 35 ± 2°C, 5 ± 2% CO_2 incubator and incubate for the desired amount of time.

12.0 RELATED FORMS

12.1 Form 3128: Virology Lab Receipt Log
12.2 Form 3129: Cell Culture Maintenance Record
12.3 Form 3131: Cell Culture Cryopreservation Record

13.0 REFERENCES

13.1 MS501: Preparation of Virus Stocks
13.2 MS502: Preparation of Cell Culture Media
13.3 Freshney, R. I., (2005). Culture of Animal Cells: A Manual of Basic Technique. (5th ed.). Hoboken, NJ: John Wiley & Sons, Inc.

14.0 MOST RECENT REVISION SUMMARY

Updated 7.10 through 7.12 to change freezing procedure to a Mr. Frosty.

Prepared by: _____ Date: 4/12/11

Quality Assurance: Sarah K Bronstad Date: 12 APR 2011

Management: Mary O Beck Date: 12 APR 2011

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ECOLAB
MICROBIOLOGICAL SERVICES

TITLE: **Preparation of Sephadex Columns**

NUMBER: **MS504-02**

EFFECTIVE: **06/01/09**

1.0 PURPOSE

To describe the preparation of Sephadex columns for use in virucidal efficacy testing.

2.0 EQUIPMENT

2.1 Balance
2.2 Biological Safety Cabinet
2.3 Pipette Filling Device (e.g. Pipetboy)
2.4 Water Bath
2.5 Refrigerator, 2 - 8°C

3.0 MATERIALS

3.1 70% Alcohol
3.2 Dulbecco's Phosphate Buffered Saline (PBS)
3.3 Bovine Albumin Fraction V
3.4 Penicillin-Streptomycin Solution
3.5 Sephadex LH-20 Beads (Sigma)
3.6 Sterile, disposable pipettes
3.7 12 mL disposable syringes
3.8 Glass wool

4.0 ASEPTIC TECHNIQUE

4.1 Aseptic technique must be practiced throughout every cell culture procedure.

5.0 PREPARATION OF SEPHADEX GEL

5.1 Add the following reagents to one 500 mL bottle of PBS:

5.1.1 Add five grams of Bovine Albumin Fraction V (for a 1% concentration).

5.1.1.1 Warm PBS in a 35°C water bath until Bovine Albumin has dissolved.

5.1.1.2 Filter sterilize the PBS using a 0.22 µm size filter unit.

5.1.2 Add five mL of Penicillin-Streptomycin solution.

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TITLE: Preparation of Sephadex Columns

NUMBER: MS504-02

5.1.3 Add 84 g of Sephadex beads.

5.2 Allow the beads to swell at 2 - 8°C for at least 12 hours before use.

5.3 Sephadex gel solution expires six months from the date of preparation.

6.0 PREPARATION OF SEPHADEX COLUMNS

6.1 Pack a small amount of sterile glass wool into a sterile syringe using a forceps. Use the plug end of a serological pipet to pack the glass wool down into the syringe. Ensure the opening in the bottom of the syringe is covered.

Note: The syringe may be packed in advance of the test date and autoclaved for later use.

6.2 Place syringes into 50 mL conical tubes. Aseptically store the plungers for use in testing.

6.3 Swirl the Sephadex gel solution well and fill each syringe to the top. Allow the excess PBS to drain.

6.4. Continue to fill the syringe with Sephadex gel solution until the bed size reached above the 12 mL mark.

6.5 Column should be stored at 2 – 8°C until use.

7.0 RECORDS STORAGE

7.1 Form 3139 from the current year will be stored in the Sephadex Gel binder.

7.2 Completed records will be archived in the first quarter of the following year. For example, records from 2009 will be archived by March of 2011. Records will be transferred to Ecolab Archives at the Ecolab Schuman Campus in Eagan, MN or to an approved off-site location.

8.0 RELATED FORMS

8.1 Form 3139: Sephadex Preparation Record

9.0 REFERENCES

9.1 ASTM E 1482: Standard Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations

TITLE: Preparation of Sephadex Columns

NUMBER: MS504-02

10.0 MOST RECENT REVISION SUMMARY

Changed ECOLAB INC. to ECOLAB in the header on the first page. Added section 6.0. Revised years listed in 7.2. Revised 9.1.

Prepared by: _____ Date: 5/12/09

Quality Assurance: _____ Date: 5/12/09

Management: _____ Date: 5/13/09

ECOLAB
MICROBIOLOGICAL SERVICES

TITLE: **Synthetic Hard Water Preparation & Standardization**

NUMBER: **MS008-23**

EFFECTIVE: **02/01/12**

1.0 PURPOSE

To describe how to prepare standardized synthetic hard water solution to be used for diluting products that possess hard water claims.

2.0 SYNTHETIC HARD WATER PREPARATION

2.1 Fill out a media preparation sheet for Solution A and Solution B. Retain in the Media Preparation Log Book.

2.2 Solution A Preparation

Magnesium Chloride ($MgCl_2 \cdot 6H_2O$)	67.74 ± .1 g
Calcium Chloride ($CaCl_2 \cdot 2H_2O$)	97.99 ± .1 g
Sterile Milli-Q Water	1 L

2.2.1 Dissolve powders in 600 mL of boiled Milli-Q water, and then bring to 1 L volume in a 1 L volumetric flask after solution has cooled.

2.2.2 Dispense into appropriate containers (for example, 250 mL Pyrex screw cap bottles) and autoclave for ≥ 15 minutes at ≥ 121°C.

2.2.3 Label using the standard Ecolab labels with a 1 month expiration date and store at 2 – 8°C.

2.2.4 Quality Control

2.2.4.1 Visual: Clear solution
2.2.4.2 Sterility Check: Sterile after incubation at 32 ± 2°C for ≥ five days
2.2.4.3 Expiration Date: One month at 2 – 8°C

2.3 Solution B Preparation

Sodium Bicarbonate ($NaHCO_3$)	56.03 ±.1 g
Sterile Milli-Q Water	1 L

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-23

2.3.1 Dissolve in 600 mL of boiled Milli-Q water, then bring to 1 L volume in a 1 L volumetric flask with Milli-Q water after solution has cooled.

2.3.2 Filter sterilize through a 0.45 micron filter into appropriate sterile containers. (approximately 150 - 200 mL per container)

2.3.3 Label using the standard Ecolab labels with a one month expiration date and store at 2 – 8°C.

2.3.4 Quality Control

2.3.4.1 Visual: Clear solution
2.3.4.2 Sterility Check: Sterile after incubation at 32 ± 2° C for ≥ five days
2.3.4.3 Expiration Date: One month at 2 – 8° C

2.4 Hard Water Preparation

2.4.1 To avoid precipitation of the hard water solution, water should be at room temperature before the addition of Solutions A or Solution B.

Total hardness as ppm $CaCO_3$ = 2.495 × ppm Ca + 4.115 × ppm Mg

2.4.2 To each 1 L of water to be prepared add 1 mL of Solution A for each 100 ppm of $CaCO_3$ hardness desired plus 4 mL of Solution B (e.g. for 500 ppm synthetic hard water add 5 mL of Solution A and 4 mL of Solution B per liter of water).

2.4.3 Bring to 1 L volume with sterile Milli-Q water. If preparing more than 1 L, combine flasks in a sterile 4 L beaker blender after adding appropriate amounts of Solutions A and Solution B and bringing to volume.

2.5 Alternate Hard Water Preparation: Commercial Preparation

2.5.1 Use a commercially available standard, preferably NIST traceable, to prepare synthetic hard water (e.g. Hach Chemical Company 218710).

2.5.2 To prepare a 400 ppm as $CaCO_3$ solution, add four ampules of 10,000 ppm as $CaCO_3$ standard (10 mL each ampule) to a 1 L volumetric flask.

2.5.3 Add sterile Milli-Q water up to 1 L mark. Solutions of other water hardness and different volumes may be prepared as appropriate.

2.6 The pH of all test waters less than 2000 ppm hardness (as $CaCO_3$) should be 7.6 – 8.0. Adjustment of hard water pH using NaOH or HCl may be necessary depending on the starting water pH.

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TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-23

3.0 STANDARDIZATION OF SYNTHETIC HARD WATER

3.1 Method Check – Prior to standardization of the synthetic hard water, the accuracy of the titration method must be checked by analyzing a 500 ppm $CaCO_3$ standard. This must be performed on a monthly basis or when testing new batches of Solutions A and Solution B.

3.1.1 Dilute 10 mL of a 1000 ppm $CaCO_3$ standard (1 mL = 1 mg $CaCO_3$) in 10 mL of Milli-Q water to result in a 500 ppm $CaCO_3$ solution.

3.1.2 Dilute 10 mL of the 500 ppm $CaCO_3$ solution in 40 mL of Milli-Q water in a beaker.

3.1.3 Test solution as described in 3.2.2 – 3.2.5.

3.1.4 The hardness of the 500 ppm solution is determined as follows:

$$\text{hardness (ppm)} = (\text{mL EDTA}) \times 100$$

3.1.5 Record the result and the lot number of the standard on Form 3011. Hardness of the 500 ppm $CaCO_3$ solution must be 500 ± 20 ppm $CaCO_3$. Failure of the standard to fall within this range indicates a problem in the test method. Corrective actions should be documented in the comments section on Form 3011. The procedure may be used for standardization of synthetic hard water only when results of the standard are within the range described above.

3.1.6 Records from the current and previous year will be kept in the Microbiological Services Equipment Maintenance binder. All earlier records will be archived in the first quarter of the current year. For example, records from 2010 will be archived by March of 2012. Records will be transferred to Ecolab Archives at the Ecolab Schuman Campus in Eagan, MN or to an approved off-site location.

3.2 Sample Testing/Standardization

3.2.1 Dilute 10 mL of prepared hard water in 40 mL of Milli-Q water in a beaker.

3.2.2 Add 1 mL water hardness buffer with magnesium. Use hood when adding; the buffer has irritating vapors.

3.2.2.1 The buffer is VWR product code VW3491 (or equivalent)

TITLE: **Synthetic Hard Water Preparation & Standardization**

NUMBER: **MS008-23**

3.2.2.2 Approximate composition of buffer, % by weight:

Ammonia	56-57
Ammonium chloride	6-7
EDTA-Magnesium Tetraacetate Salt	0.5
Water	> 35

Note: This buffer has a relatively short expiration.

3.2.3 Optional: Add 1 mL inhibitor – needed only if previous titration without it has been unsatisfactory (refer to 3.2.5.2).

3.2.4 Add just enough Ecolab hardness indicator #016 to yield a pink coloration upon dissolving.

3.2.4.1 Hardness indicator 016 contains Calgamite (1-(1- hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid) as the actual indicator, along with inert ingredients.

3.2.4.2 It is obtained from Ecolab Test Kits (order through F&B Customer Service) at the Ecolab Engineering Center.

3.2.5 Add 0.01M EDTA slowly until the pink coloration turns blue. Record the number of milliliters of EDTA needed to create the color change.

3.2.5.1 The titration should be completed within five minutes of buffer addition to minimize tendency toward $CaCO_3$ precipitation.

3.2.5.2 If the end point color change is not clear and sharp (e.g. the color changes to blue and then drifts back to pink) then an inhibitor/complexing agent must be added (or possibly, the indicator has deteriorated).

3.2.5.3 Prepare inhibitor solution by dissolving 5.0 g sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$) or 3.7 g $Na_2S \cdot 5H_2O$ in 100 mL distilled water. Prepare and dispense in hood. This inhibitor solution deteriorates quickly though air oxidation and should be made each day it is needed.

3.2.5.4 Dilute new sample of test solution and re-titrate beginning with step 3.2.2, including addition of inhibitor.

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TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-23

3.2.6 The hardness of the water is determined as follows:

Hardness as mg $CaCO_3$ / L = (mL EDTA × 1000)/10 mL of sample
= mL EDTA × 100

3.2.7 Upon titration, hardness must not exceed 20 ppm above or below the ppm *specified in test procedure/protocol/lab statement. Therefore, if a claim is* for 500 ppm, the titration must yield 500 ± 20 ppm. If ppm hardness is out of the established range, the sample should be retitrated. Upon a second titration, if ppm hardness is still outside established ranges, the hard water must be diluted or additional solution added to yield the desired ppm. After adjustments have been made, the water must be titrated to determine ppm hardness.

3.2.8 Only two adjustments may be made to the hard water following the above procedure. If the hard water is outside the established limits after two adjustments, the water must be disposed of and the process reinitiated.

3.2.7 For GLP testing, record Hard Water Preparation and Standardization on Form 3010 or Form 3113.

4.0 RELATED FORMS

4.1 Form 3010: Synthetic Hard Water Preparation & Standardization
4.2 Form 3011: Water Hardness Standard Results
4.3 Form 3072: Solution A Prep Log
4.4 Form 3074: Solution B Prep Log
4.5 Form 3113: Test Substance Use-Solution Preparation for Analysis

5.0 REFERENCES

5.1 AOAC (2011) Method 960.09 (E)
5.2 APHA, Standard Methods for the Examination of Water & Wastewater, 21st Ed., 2005. Section 3500-Ca B. EDTA Titrimetric Method.

6.0 MOST RECENT REVISION SUMMARY

Added an alternate method of preparing hard water in 2.5. Updated reference in 5.1.

Prepared by: _____ Date: 11 Jan 2012
Quality Assurance: Sherri St. Clair Date: 11 Jan 2012
Management: _____ Date: 11 Jan 2012

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MICROBIOLOGICAL SERVICES

TITLE: **Test Substance Use-Solution Preparation for Analysis**

NUMBER: **MS088-18**

EFFECTIVE: **07/02/12**

1.0 PURPOSE

To describe the preparation and active ingredient analysis of a diluted test substance (test substance use-solution). Use-solution analysis is included with pesticide efficacy studies, chemical quality verification studies and contract lab studies to verify that the active ingredient concentration corresponds to the dilution made for the claimed active ingredient concentration in the undiluted test substance.

2.0 PROCEDURE

2.1 Typically, use-solutions are prepared as follows:

2.1.1 Use-solutions prepared at the Lower Certified Limit (LCL) are for efficacy studies and Chemical Quality Verification (CQV) of the use-solution for efficacy studies of EPA regulated products

2.1.2 Use-solutions prepared at the Upper Certified Limit (UCL) are for contract lab TOX studies and CQV of the use-solution for contract lab TOX studies of EPA regulated products

2.2 Determine the concentration of active ingredient in the test substance concentrate to verify it is within claimed limits. Perform the analysis for each active ingredient in the product.

2.3 Deionized water may be used as the test substance diluent or the test substance diluent (e.g. hard/soft water or label instructed diluent) may be prepared in the same manner as used for pesticide efficacy testing.

2.4 Prepare the test substance use-solution according to label instructions or as specified in protocol using diluent as described in 2.3. This use-solution should be labeled according to M032.

Example: A 1:64 dilution is 1 part test substance, 63 parts diluent.

2.5 Analyze the test substance for active ingredient concentration using the same validated QATM that is, or will be, included in the finished good Bill of Quality (BOQ).

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6/30/12

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TITLE: **Test Substance Use-Solution Preparation for Analysis**

NUMBER: **MS088-18**

Note: The method used to measure active ingredient concentration in the use-solution may have limited sensitivity, accuracy and precision for quantitating the minimal levels of active ingredient found in many use-solutions. These factors may need to be considered when interpreting results. Any modifications to the QATM to adjust for this should be specified in the protocol.

2.6 Analyze the results. The active ingredient concentration in the use-solution should correspond to the dilution made for the claimed active ingredient concentration in the concentrate (e.g. EPA Upper & Lower Certified Limits) and to 40 CFR § 158.350 Certified Limits unless otherwise noted in the protocol. A scientific explanation must accompany any result which does not correspond to the dilution made for the claimed active ingredient level in the concentrate.

3.0 Formulas to Determine Use-solution Amounts and Acceptance Criteria

3.1. Dilution Factor (DF) Determination

3.1.1 Dilution Factor by Volume (DF_{vol})

Example: $\text{Dilution Factor } (DF_{vol}) = \left(\frac{1\text{ oz}}{1\text{ gallon}}\right)\left(\frac{1\text{ gallon}}{128\text{ oz}}\right) = 0.0078$

3.1.2 Density/Specific Gravity (SG) Calculation

Obtain density or specific gravity values from confidential statement of formula (CSF) or suitable documentation. Convert as necessary to g/mL or unitless for SG.

Conversion Example:

$$\left(\frac{9.2\text{ lbs}}{\text{gallon}}\right)\left(\frac{1\text{ gallon}}{3785.412\text{ mL}}\right)\left(\frac{453.5924\text{ g}}{1\text{ lb}}\right) = 1.102\text{ g/mL}$$

$$\text{Density of Product} = \frac{\text{mass (g)}}{\text{volume (mL)}};$$

$$\text{Specific Gravity} = \frac{\text{Density of Product}}{\text{Density of Water (1.0 g/mL)}}$$

$$\text{Density of Product} = 9.2\text{ lbs/gal} \sim 1.102\text{ g/mL};$$

$$\text{Specific Gravity} = \frac{1.102\text{ g/mL}}{1.0\text{ g/mL}} = 1.102$$

3.1.3 $DF = DF_{vol} \times SG$

$DF = 0.0078 \times 1.102 = 0.0086$

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-18

3.2. Use-solution prepared per label (e.g. 1000 g use-solution prepared at 1 oz/gallon dilution)

3.2.1 Target mass (g) of product = [Total use-solution mass (g)] ×DF

Target mass (g) of product = 1000 g × 0.0086 = 8.6 g

3.2.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Target mass (g) of diluent = 1000 g – 8.6 g = 991.4 g

3.2.3 Include a range of ± 0.03 g (~ 1 drop) or ± 0.3 g (~ 10 drops) to target masses when preparing use-solutions.

Note: any appropriate total use-solution mass may be used.

3.3. Use-solution prepared at CSF lower certified limit (LCL) – 1 active ingredient

3.3.1 Determine the active ingredient concentration (ppm) in the test substance use-solution when diluted (per label or protocol) using the test substance (concentrate) with active ingredient(s) at the LCL.

Example: 1 oz/gallon

$$\%\ \text{Dilution} = \left(\frac{1\ \text{oz Product}}{1\ \text{gallon}}\right)\left(\frac{1\ \text{gallons}}{128\ \text{oz}}\right)(100\%) = 0.781\%$$

$$\text{ppm active at LCL} = \left(\frac{\%\ \text{Active at LCL}}{100\%}\right)\left(\frac{\%\ \text{Dilution}}{100\%}\right)\left(\text{Specific Gravity} \times 10^{6}\right)$$

$$\text{Target mass (g) of product} = \frac{\text{ppm Active at LCL} \times \text{Total mass of use - solution} \times 100\%}{10^{6} \times (\%\ \text{Active Ingredient Result})}$$

3.3.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Note: any appropriate total use-solution mass may be used.

3.4. Use-solution prepared from CSF lower certified limit (LCL) – multiple active ingredients

- Ensure that all active ingredients are at or below the calculated lower acceptance limit.
- This can be determined by calculating all active ingredient amounts and using an amount (of product) that ensures all active ingredients present to be less than or equal to the calculated lower acceptance limit.

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-18

3.4.1 Follow 3.3 to determine target masses (g) of product and diluent.

Note: any appropriate total use-solution mass may be used.

3.5. Use-solution prepared at CSF upper certified limit (UCL) – 1 active ingredient

3.5.1 Follow 3.3 and replace LCL values with UCL values.

Note: any appropriate total use-solution mass may be used.

3.5.2 A use-solution can be purposefully prepared greater than the calculated ppm at UCL concentration.

3.6. Use-solution prepared at CSF upper certified limit (UCL) – multiple active ingredients

- Ensure that all active ingredients are at or above the calculated upper acceptance limit.
- This can be determined by calculating all active ingredient amounts and using an amount that ensures any active ingredient present to be greater than or equal to the calculated upper acceptance limit.

3.6.1 Follow calculations in 3.5 (replace LCL values with UCL values) to determine target masses (g) of product and diluent.

Note: any appropriate total use-solution mass may be used.

3.6.2 A use-solution with multiple actives can be purposefully prepared greater than the calculated ppm at UCL concentration.

3.7. Acceptance criteria formulas and calculations for LCL and UCL dilution use-solutions

3.7.1 **Example:** Product diluted at 1 oz/gallon (product/diluent) for LCL dilution use-solutions

Where: CSF LCL = 16.43%; DF = 0.0086; Nominal (N) = 17.29%

Lower Acceptance Level = CSF LCL × DF = 16.43% × 0.0086 = 0.141%

When the analyte of interest in the use-solution at the lower acceptance limit is ≤ 1.0% after dilution; acceptance criteria may be expanded to accommodate method variability or other suitable rationale. Expanded

TITLE: **Test Substance Use-Solution Preparation for Analysis**

NUMBER: **MS088-18**

ranges are based on 40 CFR § 158.350 (Certified Limits) for LCL dilution use-solutions.

If the nominal concentration (N) for the ingredient is	Upper/Lower Acceptance Limits after dilution may be adjusted as follows	
	Upper Limit	Lower Limit
$N \leq 1.0\%$	N + 10%	N - 10%
$1.0\% < N \leq 20.0\%$	N + 5%	N - 5%
$20.0\% < N \leq 100.0\%$	N + 3%	N - 3%

Therefore

Lower Acceptance Limit = 0.141% - 10% → [0.141% – (0.141 × 0.1)] = 0.127%
Upper Acceptance Limit = 0.141% + 10% → [0.141% + (0.141 × 0.1)] = 0.155%

Products with CSF LCL/UCL values greater than N ± 10% should follow the same range as calculated from the CSF.

Example

Lower Acceptance Limit = 0.141% - 25% → [0.141% – (0.141 × 0.25)] = 0.106%
Upper Acceptance Limit = 0.141% + 25% → [0.141% + (0.141 × 0.25)] = 0.176%

3.7.2 Example: Product diluted at 1 oz/gallon (product/diluent) diluted at UCL.

Where CSF UCL = 18.15%;DF = 0.0086;N = 17.29%

Upper Acceptance Limit = (CSF UCL × DF) = 18.15 × 0.0086 = 0.156%

The acceptance criteria may be expanded around the UCL dilution. The acceptance range may be increased by 20% and decreased by 10%

Lower Acceptance Limit = (CSF UCL × DF) – 10% → [0.156 – (0.156 × 0.1)] = 0.140%
Upper Acceptance Limit = (CSF UCL × DF) + 20% → [0.156 – (0.156 × 0.2)] = 0.187%

The acceptance criteria range may be adjusted based on protocol criteria and suitable rationale.

3.8. Acceptance criteria formulas and calculations for use-solutions diluted to the CSF LCL or UCL.

3.8.1 **Example:** Product diluted to 1 oz/gallon

Acceptance criteria for use-solutions diluted to the CSF LCL or UCL are greater than or equal to the Upper/Lower acceptance limits.

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TITLE: **Test Substance Use-Solution Preparation for Analysis**

NUMBER: **MS088-18**

Acceptance Limit (Active at CSF LCL) = CSF LCL × DF = 16.43% × 0.0086 = 0.141%
Acceptance Limit (Active at CSF UCL) = CSF UCL × DF = 18.15% × 0.0086 = 0.156%

Therefore

Acceptance Criteria (Active at CSF LCL) ≤ 0.141%
Acceptance Criteria (Active at CSF UCL) ≥ 0.156%

4.0 RELATED FORMS

4.1 Form 3113: Test Substance Use-Solution Preparation for Analysis

5.0 REFERENCES

5.1 M032: Labeling Requirements
5.2 40 CFR 158.350

6.0 MOST RECENT REVISION SUMMARY

Deleted option of preparing use-solution at label and all corresponding sections. Added CQV use-solution to 2.1.1 and 2.2.2. Added new 3.5.2 and 3.6.2 to allow use-solution to be prepared above calculated ppm at UCL. Revised 3.7.1 to give criteria formulas for use-solutions at LCL. Added 3.7.2 for criteria for use-solutions prepared at the UCL.

Prepared by: Lisa Hellickson Date: 14 June 2013
Quality Assurance: Sherri St. Clair Date: 14 Jun 2012
Management: Mary Beth Date: 14 Jun 2012

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Form-Ver. 6016-06
Effective: 06/01/11
Form Page 1 of 1

Regulated Study Protocol Amendment

Study Title:	**Aqualogic Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Virus: Rhinovirus Type 37 -260 ppm**
Study Number:	1200063
Amendment Number:	1200063-1A
Amendment Effective:	July 17, 2012

Description of Amendment

- The Exposure Time/Temperature section of the protocol is amended to correct the exposure time to 30 seconds.
- The first sentence in the Test Method Requirement and Test System Justification section of the protocol is amended to read:

  Data from one test surface for each of two different batches of test substance are required per U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2200 Disinfectants for Use on Hard Surfaces –Efficacy Data Recommendations March 12, 2012.

Scientific Basis for Amendment

For both items, the protocol is amended to correct typographical errors in the protocol.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment _____ for details.
Initial & Date _____

Brand Cook
☒ *Study Sponsor* ☐ *Divisional Representative*
Date 7/18/2012

Susan Hellinkson
☒ *Study Director* ☐ *Study Monitor*
Date 17 Jul 2012

Printed & Verified Initial & Date 17 Jul 2012 LAH

Page 1 of 1

Form-Ver. 6016-07
Effective: 09/14/12
Form Page 1 of 1

Regulated Study Protocol Amendment

Study Title:	**Aqualogic Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Virus: Rhinovirus Type 37 -260 ppm**
Study Number:	1200063
Amendment Number:	1200063-2A
Amendment Effective Date:	October 16, 2012

Description of Amendment

The last equation in the Chemical Quality Verification, B. Method, section of the protocol is amended to change LL to LCL.

Amount of Test Substance needed to be at or below the LCL =
ppm at LCL X 100 X g amount of use-solution to be made = grams of Test Substance
(% active) 10^6

Scientific Basis for Amendment

This amendment corrects a typographical error to the last equation in the Chemical Quality Verification, B. Method, section of the protocol.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment _____ for details.
Initial & Date _____

☒ *Study Sponsor* ☐ *Divisional Representative* — *Date* 10/23/2012

☒ *Study Director* ☐ *Study Monitor* — *Date* 16 Oct 2012

Printed & Verified Initial & Date AH 10/16/12

Page 1 of 1

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Form-Ver. 6016-07
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Form Page 1 of 1

Regulated Study Protocol Amendment

Study Title:	**Aqualogic Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Virus: Rhinovirus Type 37 -260 ppm**
Study Number:	1200063
Amendment Number:	1200063-3A
Amendment Effective Date:	October 19, 2012

Description of Amendment

- The Chemical Quality Verification section of the protocol is amended to list the CSF upper certified limit as 0.1031% free available chlorine as listed in the table below.

Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Free Available Chlorine*	0.0660%	0.1031%

*The equivalent weight of NaOCL (sodium hypochlorite) to the equivalent weight of Cl_2 (Chlorine) is 37.2/35.5 = 1.05. Dividing the sodium hypochlorite concentration by the ratio of the equivalent weight of sodium hypochlorite to the equivalent weight of chlorine results in the free available chlorine concentration.

- The Test Substance Concentration section of the protocol is amended to list the CSF upper certified limit as 0.1031% available chlorine as listed in the table below.

Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Available Chlorine	0.0660%	0.1031%

Scientific Basis for Amendment

The protocol was amended to update the upper certified limit to match a revised CSF. The previous value had a rounding error in the available chlorine value for the upper limit.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment _____ for details.
Initial & Date _____

☒ *Study Sponsor* ☐ *Divisional Representative* — *Date* 10/23/2012

☒ *Study Director* ☐ *Study Monitor* — *Date* 10/19/12

Printed & Verified Initial & Date UPH 10/19/12

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